# MSD® 384-Well MULTI-ARRAY® and MULTI-SPOT®

# Rat Cytokine Assays: Tissue Culture Kit

## Summary

MSD Cytokine Assays measure one to four cytokines in a 384-well MULTI-ARRAY or MULTI-SPOT plate.  The assays employ a sandwich immunoassay format where capture antibodies are coated in a single spot, or in a patterned array, on the bottom of the wells of a MULTI-ARRAY or MULTI-SPOT (Figure 1) plate.  Cytokine assays are available from MSD in single spot MULTI-ARRAY and 4-spot MULTI-SPOT 384-well plate formats. This product insert outlines an assay protocol recommended for tissue culture samples.  This insert also describes ways the user can modify these protocols to meet specific work flow or performance requirements.
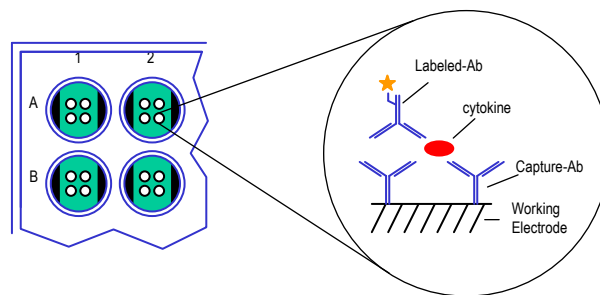
**Figure 1.**  Cytokine capture antibody is pre-coated on specific spots of a 4-Spot MSD MULTI-SPOT plate.  Calibrator solutions or samples are incubated in the MULTI-SPOT plate, and each cytokine binds to its corresponding capture antibody spot.  Cytokine levels are quantitated using a cytokine-specific Detection Antibody labeled with MSD SULFO-TAG™ reagent.

# Reagents Provided

| | | |
|---|---|---|
| **Detection Antibody Mix** | Anti-cytokine antibodies labeled with MSD SULFO-TAG reagent and pre-mixed to provide a 50 µg/mL stock solution of each antibody | 2-8 $^o$C |
| **Cytokine Calibrators** | Provided as a 1 µg/mL stock solution | ≤ -70 $^o$C |
| **Diluent 1** | RPMI-based medium for dilution of calibrators | 2-8 $^o$C |
| **Diluent 100** | Contains blocking and stabilizing agents | 2-8 $^o$C |
| **Read Buffer T** | 4X MSD Read Buffer T with surfactant | RT |
| **MULTI-ARRAY or MULTI-SPOT Plate** | 384-well MSD plate spotted with specific anti-cytokine capture antibodies | 2-8 $^o$C |

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.

# Required Materials and Equipment – not supplied

- Deionized water for diluting concentrated buffers
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions
- Phosphate-buffered saline plus 0.05% Tween-20 (PBST) for plate washing
- Appropriate liquid handling equipment for desired throughput, capable of accurately dispensing 10 to 40 $\mu L$ into a 384-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

# Sample Preparation

*This section provides a general guide for the preparation of various clinical sample types for use in MSD assays. Safe laboratory practices and personal protective equipment such as gloves, lab coat, and safety glasses should be used at all times when handling samples. All samples of a potentially infectious or hazardous origin should be handled in the manner outlined by the Center for Disease Control and the Occupational Health and Safety Administration for blood-borne pathogens and Rat and animal-source materials. When analyzing some samples, it may be necessary to dilute the sample by a factor of 2 to 10 to achieve the most accurate quantitation in the MSD Rat Cytokine Assay. Please contact MSD Customer Support with any questions.*

**Tissue Culture Supernatant**
Most tissue culture supernatant samples generally do not require any dilution prior to being used in the MSD Rat Cytokine Assay. Samples from experimental conditions with extremely high levels of cytokines may require a dilution.

**Serum and Plasma**
All solid material should be removed by centrifugation. Plasma prepared in heparin tubes commonly displays additional clotting following the thawing of the sample. Remove any additional clotted material by centrifugation. Avoid multiple freeze/thaw cycles for serum and plasma samples. Some analytes in this matrix are extremely sensitive to multiple freeze/thaw cycles and the ability to detect these analytes may decrease following the first round of thawing. Serum and plasma samples may not require any dilution prior to being used in the MSD Rat Cytokine Assay.

**Sputum**
All solid material should be removed by high-speed ultracentrifugation. The clarified sample may be extremely viscous. The MSD Rat Cytokine Serum and Plasma Assay protocol should be used for sputum samples, with increased pipetting volumes if necessary. Accurate pipetting of 10 $\mu$L may not be possible due to the sample viscosity. Samples may be used neat or diluted.

**Bronchoalveolar Lavage (BAL)**
All solid material should be removed by centrifugation. Immediately following sample collection, carrier protein (such as 1% BSA in PBS) should be added to the lavage saline to prevent loss of analyte to the labware. The calibration curve should be prepared using the same saline solution (plus carrier protein) used for sample collection. BAL samples should be tested with the MSD Rat Cytokine Serum and Plasma Assay protocol.

**Urine**
Following collection, urine may be stabilized by the addition of a concentrated stabilization buffer which is a Tris or PBS-based buffering solution containing BSA, sodium azide and protease inhibitors.
Urine Stabilization buffer consists of two components:
1)  Base Buffer Solution – It is recommended to make a 11X Base Buffer Solution, consisting of 2.2 M Tris (pH 7.6), 5.5% BSA and 0.11% Sodium azide. Store at 4$^o$C.
2)  Protease Inhibitor Solution – It is recommended to make 100X Protease Inhibitor Solution, consisting of 1.0 mg/mL AEBSF, 0.1 mg/mL aprotinin, 0.1 mg/mL pepstatin and 0.1 mg/mL leupeptin in distilled H$_2$O. Store at -20$^o$C.

Prior to use, Complete Urine Stabilization Buffer is made at 10X concentration as follows:
1)  Thaw the 100X protease inhibitor solution on ice. Add 1.1 mL of 100X Protease Inhibitor Solution to 10 mL of 11X base buffer solution.
2)  Mix. Keep 10X Complete Urine Stabilization Buffer on ice until use.

The 10X Complete Urine Stabilization Buffer should be added to the urine sample at a final concentration of 1X.

At a minimum, it is recommended to add carrier protein (such as 1% BSA in PBS) to the sample to prevent loss of analyte to the labware and minimize differences sample pH. Alternatively, samples can be directly frozen without stabilization. Store samples at -80$^o$C. In cases of 24-hour urine collection, refrigerate specimens as they are produced. When making calibrator dilutions, it is recommended that the diluent be identical to the sample matrix (in terms of pH and buffering conditions), including any diluent used for sample dilution. If necessary, the sample matrix should be immunodepleted of the protein of interest prior to the addition of the calibrators.

**Cerebrospinal Fluid**

For calibration curves, it is recommended that the calibrator diluent used for the curve preparation be identical to the sample matrix, including any diluent used for sample dilution.  If necessary, the sample matrix should be immunodepleted of the protein of interest prior to addition of the calibrators.

**Tissue and Tumor Lysates**

Lysis buffers should contain low levels of denaturing detergents (<0.1% SDS) and reducing agents (<1mM DTT).  As with other sample types, carrier protein (such as 1% BSA) should be present to prevent loss of analyte to the labware.

# Reagent Preparation

### *Prepare Calibrator and Control Solutions:*

Dilute calibrators in Diluent 1.  The calibration curve preparation instructions listed below will generate a standard curve from 10000 pg/mL to 2.4 pg/mL.  The curve should be adjusted as necessary to provide the proper range for test samples.

Diluent 1 is a standard tissue culture growth medium with 10% serum.  If tissue culture samples are in a different medium, use that medium for calibration curve preparation, however please note that if using serum-free medium, the presence of some carrier protein in solution is necessary to prevent loss of analyte to the labware.
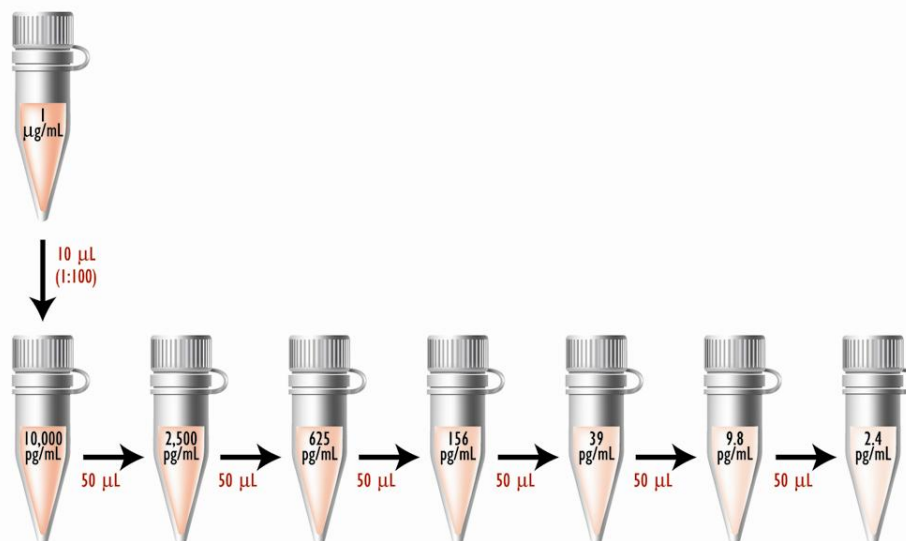
### *Prepare Calibration Curve:*

*10000 pg/mL:*       Add 10 $\mu$L of the 1 $\mu$g/mL stock solution to 990 µL of Diluent 1.  Use this high calibrator (10000 pg/mL) to prepare the standard curve following a 1:4 dilution series (as shown below).

*2500 pg/mL:*       Add 50 $\mu$L of 10000 pg/mL combined high calibrator (10000 pg/mL) to 150 $\mu$L of Diluent 1.

*625 pg/mL:*       Add 50 $\mu$L of 2500 pg/mL calibrator to 150 $\mu$L of Diluent 1.

*156 pg mL:*       Add 50 $\mu$L of 625 pg/mL calibrator to 150 $\mu$L of Diluent 1.

*39 pg/mL:*       Add 50 $\mu$L of 156 pg/mL calibrator to 150 $\mu$L of Diluent 1.

*9.8 pg/mL:*       Add 50 $\mu$L of 39 pg/mL calibrator to 150 $\mu$L of Diluent 1.

*2.4 pg/mL:*       Add 50 $\mu$L of 9.8 pg/mL calibrator to 150 $\mu$L of Diluent 1.

*0 pg/mL:*       150 $\mu$L of Diluent 1

Alternatively, calibrators can be prepared in the sample matrix or diluent of choice to verify acceptable performance in these matrices.  In general, the presence of some protein in the sample matrix is helpful for preventing loss of analyte by adsorption onto the sides of tubes, pipette tips, and other surfaces.

**Figure 2.** Calibration curve preparation from stock solution (1 μg/mL).

## Prepare Detection Antibody Solution:

Detection antibody solutions should be kept in the dark as some antibodies may be light sensitive.

The detection antibodies are provided premixed in solution at a concentration of 50 μg/mL. The working detection antibody solution should be prepared at 1.0 μg/mL. For each plate used, dilute a 160 μL aliquot of the stock Detection Antibody Mix with 7.84 mL of Diluent 100.

## Prepare Read Buffer:

The Read Buffer should be diluted 1 in 2 in deionized water to make a final concentration of 2X Read Buffer T. Add 8.5 mL of stock Read Buffer T (4X) to 8.5 mL of deionized water for each plate.

## Prepare MSD Plate:

This plate has been pre-coated with capture antibodies for specific cytokines of interest. The plate can be used as delivered; no additional preparation (e.g. pre-wetting) is required. The plate has also been exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. The plate has not been pre-blocked, and a discrete block step is only required for the specified MSD cytokine assays (as noted in the protocol).

# Protocol

1. ***Addition of Sample or Calibrator:*** Dispense 10 µL of each Calibrator or Sample Solution into a separate well of the MSD plate. Figure 3 illustrates one plate arrangement of calibrator solutions that can be used to evaluate the performance of the assay. Seal the plate with an adhesive plate seal and incubate for 1 to 2 hours with vigorous shaking (300-1000 rpm) at room temperature. In general, shaking the plate results in better mixing and more rapid binding of the sample to the capture antibodies. If a protocol without shaking is preferred, a longer incubation time (4 hours or longer) may be required to achieve the same sensitivity.

2. ***Addition of Detection Antibody Solution:*** Dispense 10 µL of the 1.0 µg/mL Detection Antibody Solution into each well of the MSD plate. Seal the plate and incubate for 1-2 hours with vigorous shaking (300-1000 rpm) at room temperature. If a protocol without shaking is preferred, a longer incubation time may be required to achieve the same sensitivity.

3. ***Wash and Read:*** Wash the plate 3 times with PBS + 0.05% Tween-20. Add 35 µL of 2X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR® Imager reader. Plates may be read immediately after addition of Read Buffer. Note: Bubbles in the fluid will interfere with reliable reading of the MULTI-ARRY and MULTI-SPOT plates. Use reverse pipetting techniques to insure bubbles are not created when dispensing the Read Buffer.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 10,000 pg/mL | | | | | | | | | | | | | | | | | | | | | | | |
| B | | | | | | | | | | | | | | | | | | | | | | | | |
| C | 2500 pg/mL | | | | | | | | | | | | | | | | | | | | | | | |
| D | | | | | | | | | | | | | | | | | | | | | | | | |
| E | 625 pg/mL | | | | | | | | | | | | | | | | | | | | | | | |
| F | | | | | | | | | | | | | | | | | | | | | | | | |
| G | 2.4 pg/mL | | | | | | | | | | | | | | | | | | | | | | | |
| H | | | | | | | | | | | | | | | | | | | | | | | | |
| I | 39 pg/mL | | | | | | | | | | | | | | | | | | | | | | | |
| J | | | | | | | | | | | | | | | | | | | | | | | | |
| K | 9.8 pg/mL | | | | | | | | | | | | | | | | | | | | | | | |
| L | | | | | | | | | | | | | | | | | | | | | | | | |
| M | 2.4 pg/mL | | | | | | | | | | | | | | | | | | | | | | | |
| N | | | | | | | | | | | | | | | | | | | | | | | | |
| O | 0 pg/mL | | | | | | | | | | | | | | | | | | | | | | | |
| P | | | | | | | | | | | | | | | | | | | | | | | | |

**Figure 3.** Suggested plate setup for calibrating Cytokine Assay Kits. Columns 1-2 contain a standard curve in duplicate. The remaining wells are available for samples. Some assays may require lower limits of detection, and in these cases, the 625 pg/mL point can be removed and a 0.6 pg/mL point added between 2.4 pg/mL and 0 pg/mL to provide an additional lower calibration point. An alternative setup that contains a larger dynamic range for the standard curve can be prepared by running calibrators in a 12-point titration in duplicate across the top or bottom of the plate. The concentrations of calibrators run may be adjusted depending on the desired dynamic range for the experiment. The data from this calibration curve can be analyzed using any standard data analysis packages.

# Additional Notes

1. ***Unwashed Assay:***  The protocol can be converted to an unwashed assay by eliminating the wash step following the detection antibody incubation, adding 15 µL **2X Read Buffer P[1]**, and analyzing the plate on the SECTOR instrument.  The use of this protocol may result in a loss of sensitivity, the effect of which will vary for each cytokine.

2. ***Sample Matrices:***  This kit is optimized for analysis of tissue culture supernatant samples.  In general, these plates have been found to work well to measure cytokine levels in a wide range of samples, including cell lysates, serum and plasma samples, and simple buffers.  Sample dilution and/or spike recovery studies in the sample matrix of interest should be carried out to verify acceptable performance in the matrix.

3. ***Combining the Sample and Detection Antibody Addition Steps:***  The protocols described above call for incubating the sample in the wells prior to the addition of the detection antibody (sequential incubations).  This procedure is used because in some selected assays using a polyclonal detection, the detection antibody itself may include antibodies that compete with the immobilized capture antibody.  In some assays, however, MSD has found that the sample and detection antibodies may be added concurrently (simultaneous incubation) with little or no loss in performance.

4. ***Stability of Assay in Read Buffer:***  The plates do not need to be read immediately after addition of Read Buffer. In the washed assay, the loss of signal is typically less than 20% over a 1-hour incubation in Read Buffer, and is usually stabilized by 2 hours. The observed loss is due to a re-establishment of equilibrium in the well.

5. ***Optimizing Assay Sensitivity:***  The washed protocols described above were developed to provide excellent assay performance with a minimum number of steps, minimal sample volume requirement, and a rapid assay completion time.  One or more of the following assay modifications may be used to further improve the assay sensitivity:

    a. *Increasing incubation times* - The incubation time with detection antibody can be increased to 4 hours with shaking (or at least 12 hours without shaking).

    b. *Increasing sample volumes up to 20 µL* (Note:  Efficient shaking and/or longer incubation times become more important at higher volumes) - If a larger sample volume is used, the concentration of detection antibody must be adjusted in the detection antibody solution to compensate for a change in volume.

---

[1] Read Buffer P is not included with this kit.  To receive a free sample of Read Buffer P, contact Customer Service (customerservice@mesoscale.com).

c. *Washing sample from the well prior to the addition of the detection antibody mix* (especially useful for large sample volumes to avoid dilution of the detection antibodies) - Not all cytokines will see an improvement by addition of this step, and some cytokines may see decreased sensitivity.

## Topics of Interest

1. ***Background signal and negative signals:*** The output signal produced by electrochemiluminescence assays is in units of counts of light measured by a charge-coupled device (CCD) camera or photodiode. As with any measurement technique, there is a certain amount of normal variation in this signal (instrument noise) which sets the threshold for the lowest levels of signal that can be measured (noise floor). This variation is different depending upon the size of the working electrode with typical values of about 10 counts for 384-well single spot plates and 50 counts for 384-well 4-spot plates. When the background signal of an assay approaches the noise floor (i.e. the mean signal of negative controls or sample blanks is close to zero), it is possible to observe negative counts for some wells.

2. ***Signal Levels:*** The camera system is linear over nearly a 6 log-dynamic range. The highest achievable signals on the SECTOR Imager 6000 and 2400 are between 1.0 and 2.0 million counts. If the signals from the highest point on the calibration curve are not approaching 1.0 million counts, the high end of the calibration curve may be extended. The lowest observed signals using the Read Buffer T (2X) are between 10 and 50. Negative signal values may occur due to instrument noise, omission or usage of the incorrect Read Buffer, or incorrect amount of detection antibody.

3. ***Fitting methods:*** To utilize the quantitative value of electrochemiluminescent detection, a titration curve is produced using a known standard. The standard curve is modeled using least squares fitting algorithms so that signals from samples with known levels of the analyte of interest can be used to calculate the concentration of analyte in the sample. MSD's cytokine assays have a wide dynamic range (typically 3-5 logs) which allows accurate quantitation in many samples without the need to dilute prior to running the assay. MSD recommends using software to fit the data that utilizes a 4-parameter logistic model (or sigmoidal dose-response) and includes a $1/Y^2$ weighting function. The weighting functionality is important because it provides a better fit of data over a wide dynamic range, particularly at the low end of the standard curve. An alternative analysis approach is to subtract the background signal from all data points, and then use a linear model to fit the data. The disadvantage of this approach is that a skewed calibration curve may be created if the background signal used for subtraction

is not an appropriate indicator of background signal over the complete curve. Also, negative numbers may be produced if background-corrected signal values are less than the instrument noise observed in signals at the low end of the curve.

4. ***Antibody pairs and cross-reactivity:*** The capture and detection antibody pairs used in MSD cytokine assays have been selected by an optimization process that is designed to minimize cross-reactivity with other cytokine assays. In addition, the chosen antibodies are specific for the particular Rat cytokine of interest and show minimal cross-reactivity with other species.

5. ***Reverse pipetting:*** Most manual hand pipets have two plunger positions for pipetting liquids. The first position is calibrated to allow aspiration and dispensing of user-specified amounts of liquid and the second (blow-out) position enables the user to expel any residual liquid after the pipet has been pushed to the first position. When a pipet is used to dispense liquid by moving the plunger to the first position followed by the second (blow-out) position, bubbles may be created in the dispensed liquid. The reverse pipetting technique is designed to allow precise pipetting while avoiding the creation of bubbles. The technique is to push the pipet plunger past the first position to the second position prior to aspirating liquid into the tip, thereby aspirating slightly more liquid than the desired volume (overdraw). In order to dispense the liquid from the tip, the pipet plunger is pushed to the first position only. This allows precise dispensing without the introduction of bubbles. When using the reverse pipetting technique, it is important not to overdraw excess liquid into the pipet mechanism.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.

# MSD® 384-Well MULTI-ARRAY® and MULTI-SPOT®

# Rat Cytokine Assays: Tissue Culture Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the MSD Rat Cytokine Assay.

**STEP 1:** **Sample and Reagent Preparation**

Prepare calibrator solutions and calibration curve.

- Use the 1 μg/mL calibrator stock to prepare an 8-point calibration curve of 10000, 2500, 625, 156, 39, 9.8, 2.4, and 0 pg/mL. The calibration curve can be modified as necessary to meet specific assay requirements.

Prepare Detection Antibody Solution by diluting the Detection Antibody to 1.0 μg/mL in 8.0 mL (per plate) of Diluent 100.

Prepare 17 mL of 2X Read Buffer T by diluting 4X Read Buffer T 1 in 2 with deonized water.

**STEP 2:** **Add Sample or Calibrator**

Dispense 10 μL/well Calibrator or Sample.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 1-2 hours.

**STEP 3:** **Add Detection Antibody**

Dispense 10 μL/well 1 μg/mL Detection Antibody Solution.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 1-2 hours.

**STEP 4:** **Wash and Read Plate**

Wash plate 3 times with PBS + 0.05% Tween-20.

Dispense 35 μL/well 2X Read Buffer T.

Analyze plate on SECTOR instrument.